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13. ABSTRACT (Maximum 200 Words)

We have previously identified the Ras-like GTPases, RalA and RalB as linchpin modulators of human tumor cell proliferation and survival. Using RNAi-mediated loss-of-function analysis, we find that RalA is critical for anchorage-independent growth whereas RalB is required for tumor cell survival. The collaboration of these isoform-specific contributions generates a minimal oncogenic platform, which results in aberrant proliferation coupled with suppression of apoptosis. We have begun to characterize the molecular basis of the collaborative contributions of RalA and RalB to cell regulation. These studies have surprisingly revealed that RalB and Sec5 are mediating tumor cell survival whereas RalA and STAT3 contribute to anchorage-independent proliferation. Understanding how the regulation occurs will be the focus of my future work.

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Introduction

We have previously identified RalA and RalB as linchpin modulators of human tumor cell proliferation and survival (Chien and White, 2003). Using RNAi-mediated loss-of-function analysis, we found that RalA is critical for anchorage-independent growth whereas RalB is required for tumor cell survival. The collaboration of these isoform-specific contributions generates a minimal oncogenic platform, which results in aberrant proliferation coupled with suppression of apoptosis.

Several Ral direct binding effectors and downstream signaling pathways have been identified. Active phospholipase D1 (PLD1) (Jiang et al., 1995; Luo et al., 1998), Ral-binding protein 1 (RalBP1) (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995), filamin (Ohta et al., 1999), and the exocyst components, Sec5 and Exo84 (Moskalenko et al., 2002; Sugihara et al., 2002; Moskalenko et al., 2003) have been identified as Ral-interacting proteins and may function as Ral effectors. PLD1 is constitutively associated with Ral proteins in cells (Jiang et al., 1995). However, activation of Ral cooperates with ADP ribosylation factor (ARF) GTPases to activate PLD1, perhaps by contributing to the formation of a PLD1 activation complex (Luo et al., 1998). Unlike PLD1, RalBP1 associates with Ral in a GTP-dependent manner (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). Several observations suggest that both Ral and RalBP1 can regulate receptor endocytosis (Nakashima et al., 1999; Jullien-Flores et al., 2000). Filamin selectively interacts with GTP-bound Ral and has been reported to mediate Ral-induced changes in cytoskeleton dynamics (Ohta et al., 1999; Sugihara et al., 2002). Finally, recent observations suggest that Ral proteins can regulate secretory vesicle trafficking through association with Sec5

and Exo84, components of the exocyst complex (Moskalenko et al., 2002; Moskalenko et al., 2003).

Expression of the constitutively active Ral mutant is sufficient to regulate several signaling events, which may contribute to cell proliferation and survival. For example, expression of the *Drosophila* constitutively active Ral mutant in S2 cells has been shown to inhibit sorbital-induced JNK phosphorylation (Sawamoto et al., 1999). However, expression of the constitutively active RalGEF mutant, which activates Ral GTPases, induces the JNK substrate, c-Jun phosphorylation in A14 cells (De Ruiter et al., 2000). It is presently unclear if Ral GTPases play a positive or negative role in the regulation of the JNK pathway in human cells. The highly controversial role of the JNK pathway in apoptosis suggests that this signaling pathway can be either pro-apoptotic or anti-apoptotic depending on the cell types and stimuli (Lin, 2003). In addition, expression of the constitutively active Ral mutant in NIH 3T3 cells is sufficient to increase the NF-kB dependent gene transcription, including the induction of cyclin D1 expression (Henry et al., 2000). The critical roles of NF-kB and cyclin D1 in cell survival and cell cycle control provide a possible mechanism for the contributions of Ral proteins to cell regulation. Finally, expression of the constitutively active Ral mutant is also sufficient to increase the phosphorylation of the transcription factor, STAT3 (Goi et al., 2000). STAT3 is constitutively active in a number of human cancers and possesses oncogenic potential (Bromberg et al., 1999).

Here, we apply a combination of loss-of-function analysis and complementation assays to identify downstream effectors and signaling pathways mediating the distinct contributions of RalA and RalB to human cancer cell proliferation and survival. Among

the direct binding effectors of Ral GTPases, we found that Sec5 is required to prevent transformed cells from initiating programmed cell death, mimicking the observations made with RalB siRNAs. While RalA expression is required to inhibit hyper-phosphorylation of endogenous JNK1/2 and its substrate, c-Jun, inhibiting JNK1 expression is insufficient to rescue the apoptotic phenotype caused by loss of RalB. In stead, we found that inhibiting STAT3 expression rescues loss of RalB induced cell death. Moreover, STAT3 expression is also required for tumor cell anchorage-independent growth, similar to the observations made with loss of RalA. Important, RalA is required and sufficient to increase STAT3 phosphorylation and nuclear translocation. These results together suggest that STAT3 mediates the anchorage-independent proliferation signal provided by RalA in tumor cells.

Body

To identify effectors and downstream signaling events mediating the contributions of Ral GTPases to human cancer cell survival and proliferation, we started with siRNA-mediated loss-of-function analysis to directly examine the consequences of inhibiting the expression of two Ral effectors, Sec5 and RalBP1, on normal and tumorigenic cell survival. As shown in the upper panel of Figure 1, we can effectively inhibit the expression of endogenous Sec5 in several human cell lines including HeLa, SW480, and HME-hTERT cells. Inhibiting Sec5 expression mimics inhibiting RalB expression, both induce cell death selectively in tumor cell lines (Figure 1), whereas inhibiting RalBP1 expression has no effect on cell survival (data not shown). In addition, FACS analysis also indicated that knocking down Sec5 protein expression in HME:hTERT cells can sensitize these cells to apoptosis in suspended cultures, as seen in the rise of the apoptotic shoulder on the FACS graph in the middle panel of Figure 1 (approximately 9% of cells were apoptotic in control group versus 35% in Sec5 knock down group).

To further test if Sec5 is the effector downstream of RalB mediating the tumor cell survival signal, we examined if RalA RNAi can rescue cell death upon loss of Sec5 expression in human tumor cell lines. Importantly, inhibiting the expression of RalA together with Sec5 rescued the apoptosis phenotype, as shown in Figure 2. These results suggest that Sec5 is the effector mediating RalB survival pathway in tumor cells. Notably, we also observed an induction of apoptosis when we inhibited the expression of DNA-dependent protein kinase (DNAPK), which has been shown to repair DNA double strand breaks, and co-inhibition of RalA expression together with DNAPK did not rescue

the cell death phenotype caused by loss of DNAPK (data not shown). This data suggests that the rescue of cell death by co-inhibition of RalA and Sec5 is selective. We wonder if the apoptotic phenotype caused by loss of Sec5 expression is due to inhibiting the exocyst complex formation. However, no apparent cell death or loss of cell number was observed when we inhibited two other exocyst subunits, Sec6 and Exo84 expression with siRNAs in tumor cell lines (data not shown). These data suggest that not all of the exocyst subunits are required for tumor cell survival. Each subunit may have its own unique function.

We took the same RNAi-based "candidate approach" to identify downstream signaling pathways mediating the contribution of RalA to anchorage-independent proliferation. We first investigated the contribution of RalA to the JNK pathway signaling through examining the consequences of inhibiting RalA expression on the phosphorylation of JNK1/2 and its substrate, c-Jun. Consistent with the observations made in *Drosophila*, inhibiting endogenous RalA expression increases the basal level of JNK1/2 activation and phosphorylation of c-Jun (Figure 3), suggesting RalA expression is required to suppress hyper-phosphorylation of the JNK pathway. However, inhibiting endogenous JNK1 expression is unable to rescue the apoptosis phenotype caused by loss of RalB in tumor cells, implying that activation of the JNK pathway upon loss of RalA is not responsible for driving cells to undergo apoptosis (data not shown).

Inhibiting the expression of endogenous STAT3 showed no apparent effect on tumor cell survival (Figure 4). However, inhibiting STAT3 expression together with RalB decreased the apoptosis phenotype caused by loss of RalB in tumor cell lines, as shown in Figure 4. This result suggests that STAT3 may function in the same signaling

pathway downstream of RalA. To test this hypothesis, we examined the requirement of STAT3 expression for anchorage-independent proliferation of tumor cells. As shown in Figure 5, inhibiting STAT3 expression with siRNAs in tumor cells reduced their anchorage-independent growth as measured by BrdU incorporation. Importantly, loss of STAT3 showed a similar extent of inhibition of anchorage-independent proliferation as inhibiting RalA expression. No additive effect was observed when we inhibited both RalA and STAT3 expression. This suggests that RalA and STAT3 may function in the same signal transduction pathway regulating anchorage-independent proliferation in tumor cells.

Consistent with the results above, we observed a decrease in steady-state STAT3 phosphorylation when we inhibited RalA expression in tumor cells (Figure 6). Moreover, expression of either the constitutively active or the fast-exchange Ral mutant (both have been shown to preferentially bind GTP) is sufficient to induce STAT3 phosphorylation (Figure 6) and subsequent translocation to the nucleus (data not shown). These findings together suggest that RalA contributes to anchorage-independent proliferation through regulating STAT3 phosphorylation and activation.

Effector domain mutants of oncogenic Ras have been successfully applied to dissect the downstream signaling pathways mediating Ras functions. We adopted the same strategy with Ral mutants to identify the signaling pathways involved in activating STAT3 phosphorylation. As shown in Figure 7, three effector domain mutants of constitutively active Ral have been generated. Each effector domain mutant is uncoupled from binding to one identified Ral effector, as shown in the table. By over-expressing these effector domain mutants in cells, we found only Ral23V,49N mutant, which is

unable to bind RalBP1, is defective in stimulating STAT3 phosphorylation (Figure 7). However, inhibiting RalBP1 expression in tumor cells with siRNAs showed no effect on steady-state STAT3 phosphorylation, as shown in Figure 7. This result suggests that there is yet-to-be identified effector downstream of Ral mediating STAT3 activation.

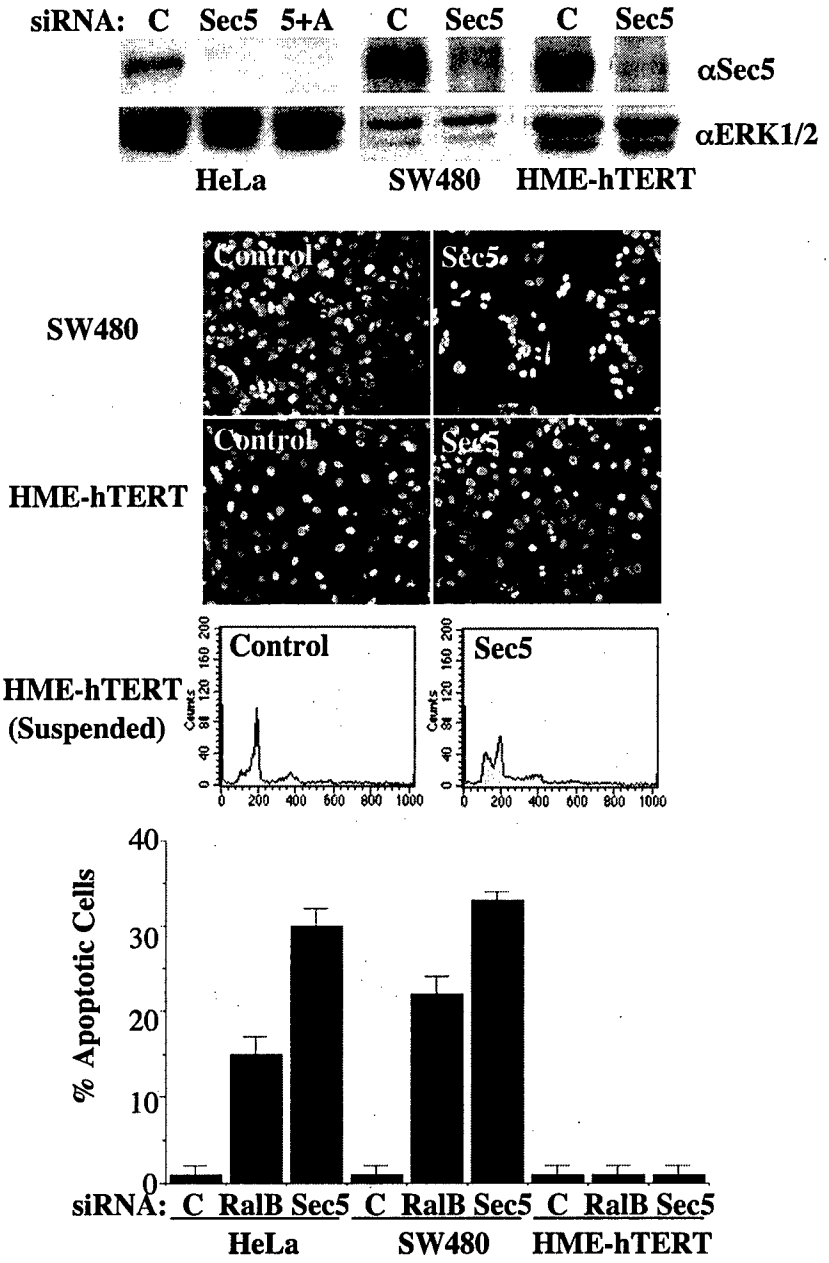

Figure 1. Sec5 mediates tumor cell survival. The indicated cell lines were transfected with siRNAs targeting control (C), or Sec5, or Sec5 and RalA (5+A) together in the upper panel. The selective inhibition of Sec5 expression was analyzed by Western blotting. 72 hours post transfection, cells were either stained with DAPI to visualize chromatin structure or analyzed by FACS sorting, as shown in the middle panel. The percent of apoptosis in the lower panels was measured by XTT assay in accordance with manufacturer's instructions.

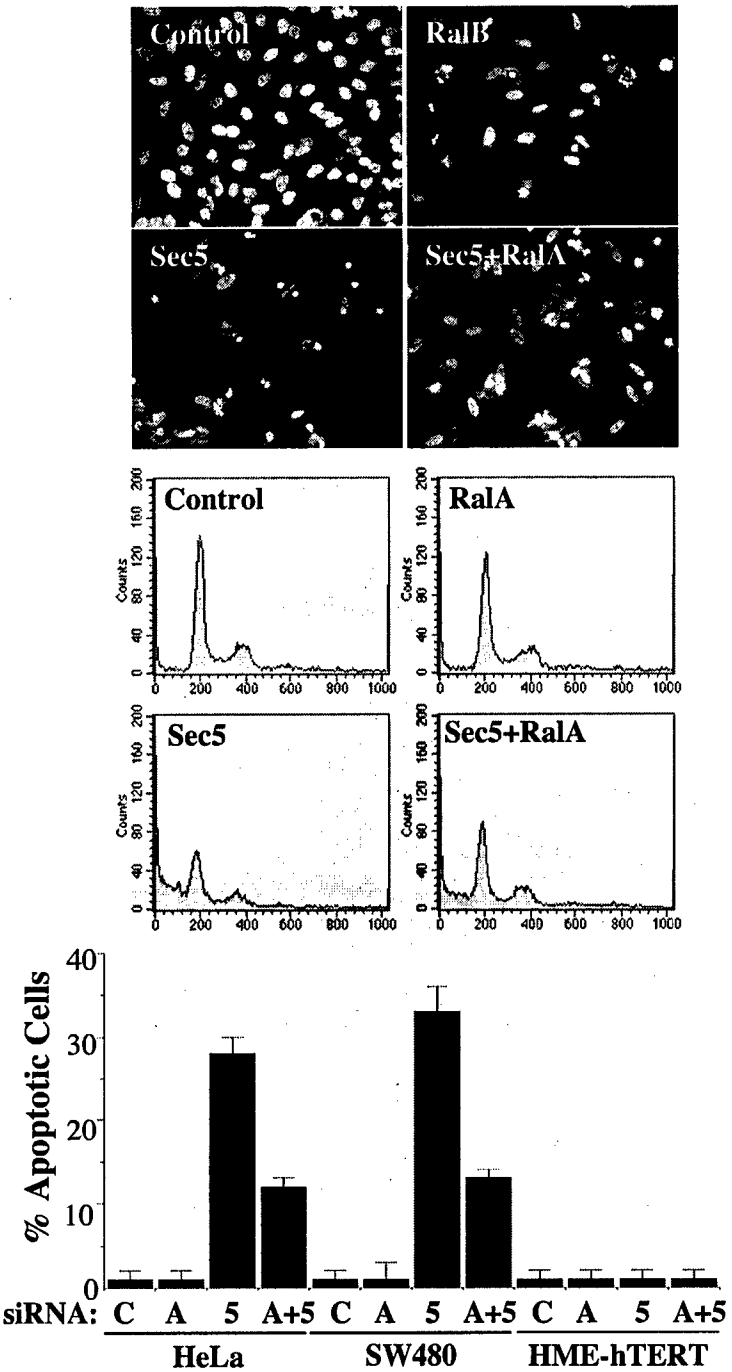

Figure 2. RalA RNAi rescues cell death upon loss of Sec5. HeLa cells were transfected with the indicated siRNAs. 72 hours post transfection, cells were either stained with DAPI to visualize chromatin structure (the upper panel) or analyzed by FACS sorting (the middle panel). The indicated cell lines were transfected with control (C), RalA (A), Sec5 (5), or RalA and Sec5 (A+5) siRNAs together in the lower panel. The percent of apoptosis was measured by XTT assay in accordance with manufacturer's instructions.

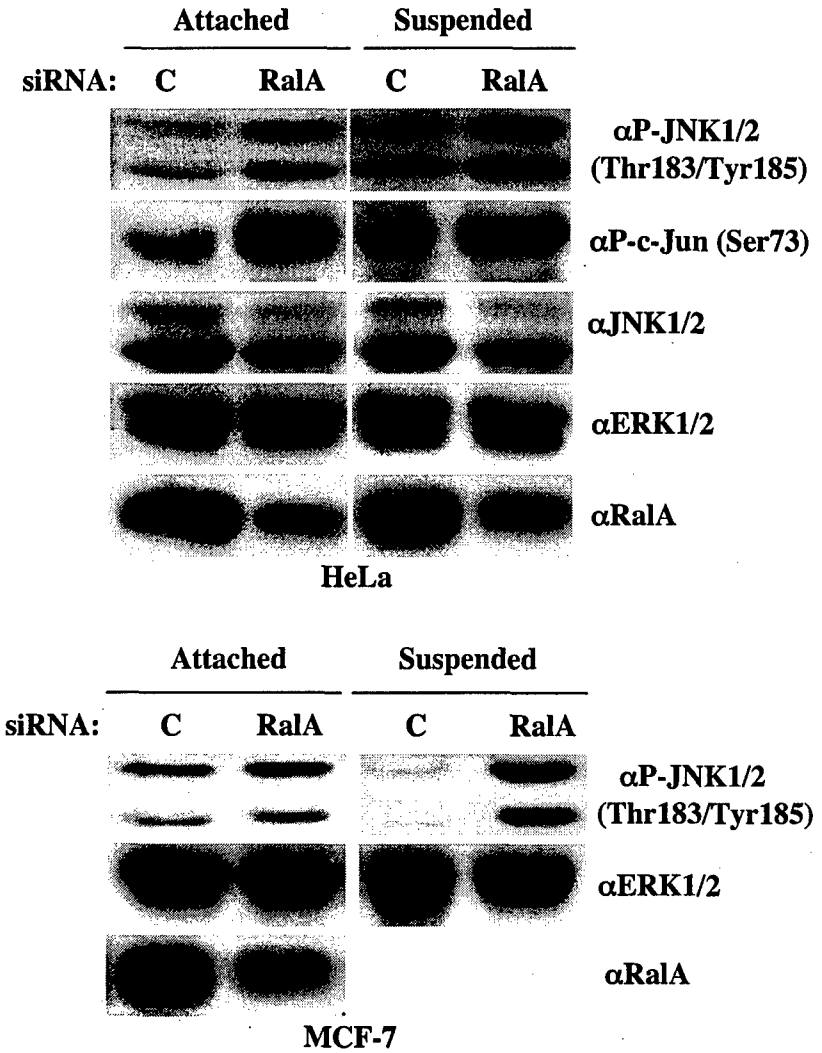

Figure 3. Inhibiting RalA expression increases basal level JNK phosphorylation. HeLa and MCF-7 cells were transfected with control or RalA siRNAs. 72 hours post-transfection, whole cell lysates of attached cells, referred to as "attached" in the figure, or cells detached from plates with trypsin and maintained in suspension for 24 hours (referred to as "suspended" in the figure) were prepared. Equivalent total proteins were analyzed by SDS-PAGE for the indicated proteins. JNK1/2 and ERK1/2 were shown as loading controls.

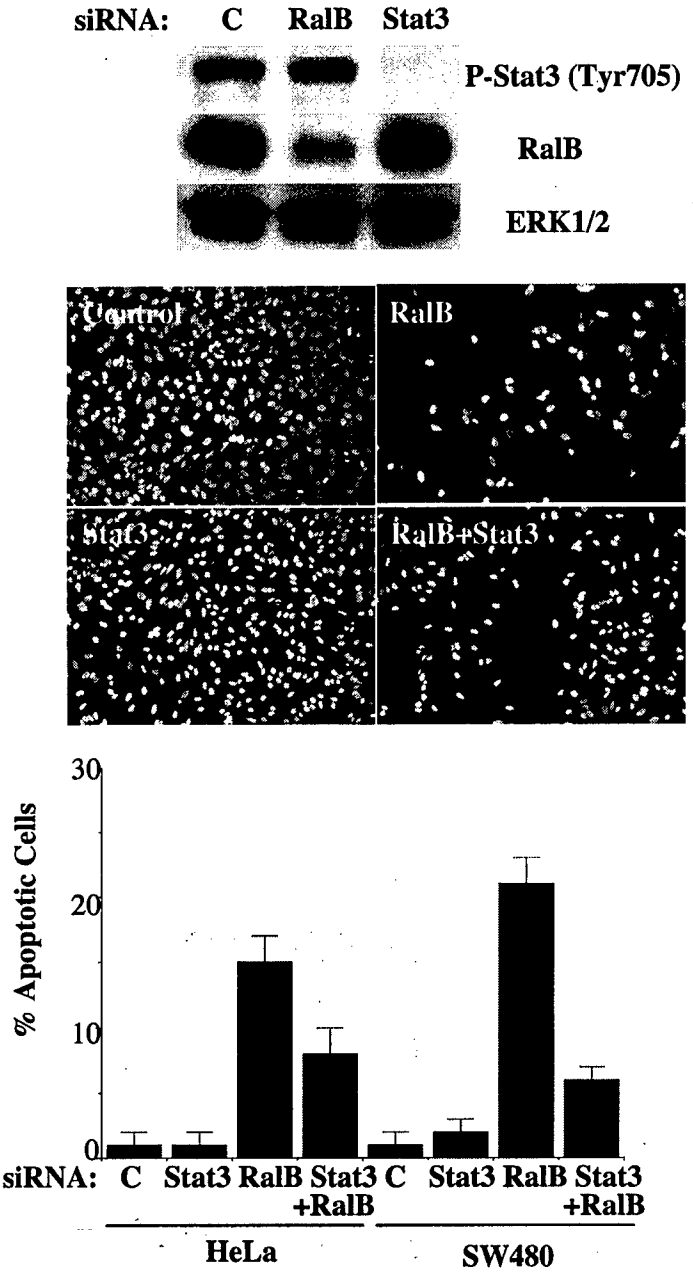

Figure 4. STAT3 RNAi rescues cell death caused by loss of RalB. HeLa cells were transfected with siRNAs targeting RalB or STAT3 alone or together. 72 hours post transfection, whole cell lysates were prepared and subjected to Western blotting (the upper panel), and cells were stained with DAPI to visualize chromatin structure (the middle panel). The percent of apoptosis in the lower panel was measured by XTT assay in accordance with manufacturer's instructions.

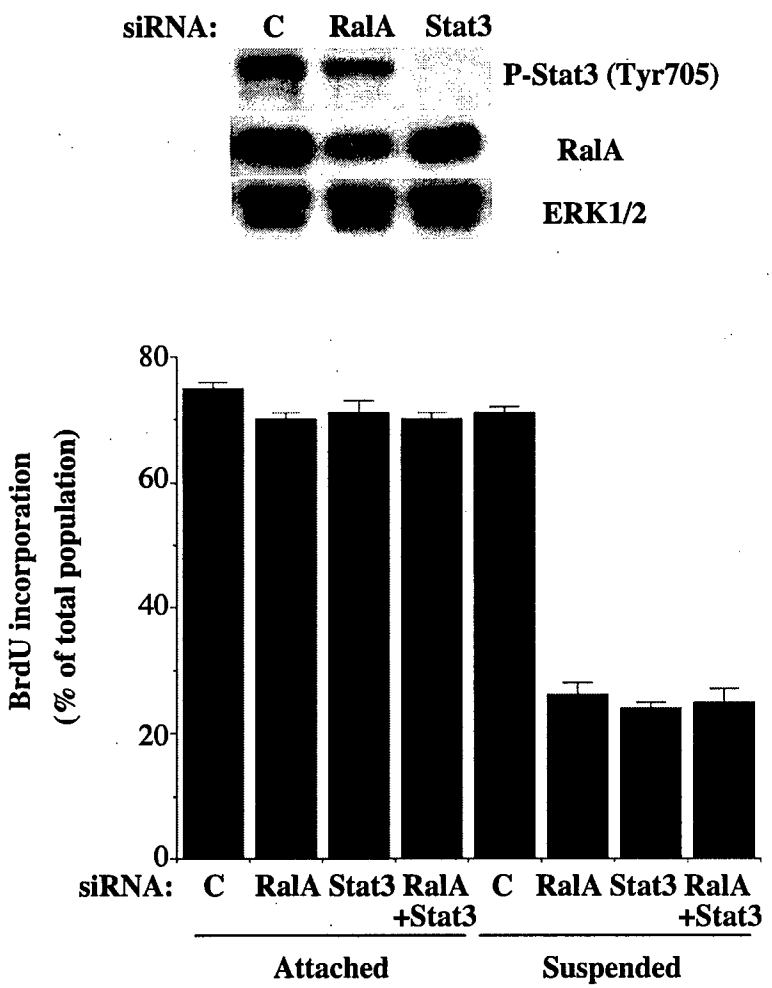

Figure 5. STAT3 mediates anchorage-independent proliferation. SW480 cells were transfected with siRNAs targeting RalB or STAT3 alone or together. 72 hours post-transfection, whole cell lysates were prepared and subjected to Western blotting (the upper panel). Proliferation assay in the lower panel was scored 48 hours post-transfection with the indicated siRNAs and GFP followed by additional 24 hours incubation with BrdU in adherent (attached) or suspension cultures.

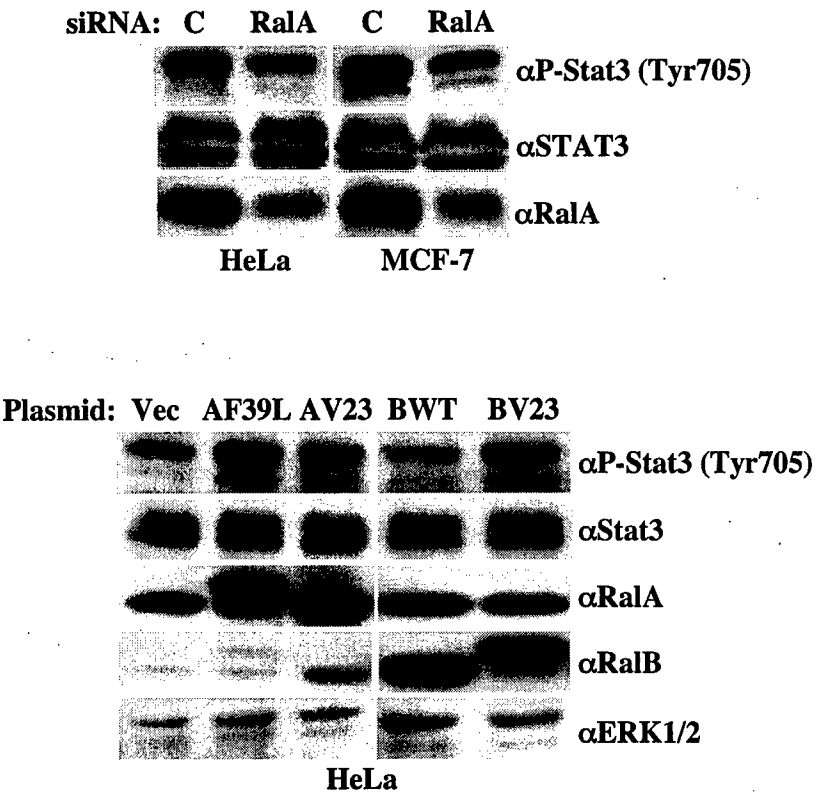

Figure 6. RalA is required and sufficient for STAT3 phosphorylation. The indicated cell lines were transfected with siRNAs targeting control (C) or RalA. 72 hours post-transfection, whole cell lysates were prepared and subjected to Western blotting (the upper panel). In the lower panel, HeLa cells were transfected with indicated plasmids. 48 hours post-transfection, whole cell lysates were prepared and analyzed with Western blotting.

	Active PLD1	RalBP1	Sec5&Exo84
RalF39L	+	+	+
Ral23V	+	+	+
Ral28N	-	-	-
Ral23V, ΔN11	-	+	+
Ral23V, 49N	+	-	+
Ral23V, 49E	+	+	-

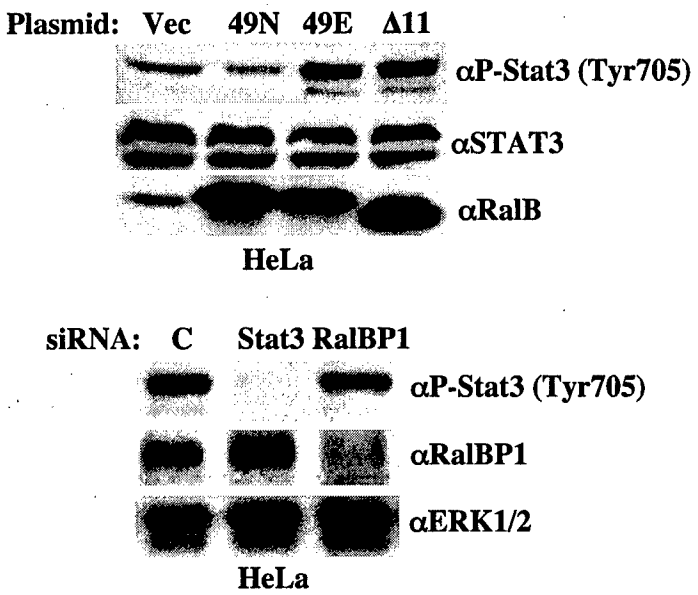

Figure 7. Ral effector domain mutants. The table above shows different binding affinity of Ral mutants toward its effectors. In the middle panel, HeLa cells were transfected with each Ral effector domain mutant. 48 hours post-transfection, whole cell lysates were prepared and subjected to Western blotting with the indicated antibodies. In the lower panel, HeLa cells were transfected with the indicated siRNAs targeting control, stat3, and RalBP1 respectively. 72 hours post-transfection, whole cell lysates were prepared and subjected to Western blotting with the indicated antibodies.

Key Research Accomplishment

Task 1. Identify RalB effectors mediating survival of breast cancer cell lines.

- Sec5 mediates tumor cell survival
- RalA RNAi rescues cell death upon loss of Sec5
- Sec5 is the effector mediating RalB-dependent survival signals in tumor cell lines

Task 2. Identify RalA effectors mediating anchorage-independent growth of breast cancer cell lines.

- STAT3 RNAi rescues cell death caused by loss of RalB
- STAT3 mediates anchorage-independent proliferation
- RalA is required and sufficient for STAT3 phosphorylation

Task 3. Define Ral GTPase as a potential breast cancer therapeutic target *in vivo*.

The animal studies described in this grant do not begin until month 24 of the study (see Statement of Work Task 3).

Reportable Outcomes

Publications

1. Balakireva, M., Rosse, C., **Chien, Y.**, Gonzy-Treboul, G., Gho, M., Voegeling-Lemaire, S., Aresta, S., White, M. A. Lepesant, J., and Camonis, J (2004). Ral supports cell fate determination by countering apoptotic programs via the JNK pathway. In Review.

2. **Chien, Y.**, Zhao Y, and White, M. A. (2005). Molecular basis of Ral GTPases support of tumorigenic regulatory pathways. In Preparation.

Invited Seminar

Proteomic Dissection of RalB-Sec5-NF-kB Pathway. (Oral Presentation) 53rd American Society for Mass Spectrometry (ASMS) Annual Conference, June 2005.

Conclusions

We have begun to characterize the molecular basis of the collaborative contributions of RalA and RalB to cell regulation. Several Ral-interacting proteins have been identified that may mediate Ral function in cells. These include phospholipase D1 (PLD1) (Jiang et al., 1995; Luo et al., 1998), Ral-binding protein 1 (RalBP1) (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995), filamin (Ohta et al., 1999), and the exocyst subunits Sec5 and Exo84 (Moskalenko et al., 2002; Sugihara et al., 2002; Moskalenko et al., 2003). We are using RNAi-mediated loss-of-function analysis to directly examine the contribution of these Ral effectors to Ral functions in normal and tumorigenic epithelial cells. These studies have surprisingly revealed that among these effectors, Sec5 mediates RalB-regulated survival pathways. Inhibiting Sec5 expression mimics inhibiting RalB expression. Loss of Sec5 is selectively toxic to tumor cell lines, and this toxicity can be reversed upon inhibiting RalA expression.

We took the same RNAi-based "candidate approach" to identify Ral effectors mediating the contribution of RalA to anchorage-independent proliferation. We found that STAT3, one of a key signaling molecule regulating transcription in response to many cytokines and growth factors, can be regulated by Ral GTPases. Inhibiting RalA expression decreases STAT3 phosphorylation selectively in tumor cell lines. In addition, over-expressing constitutively active or fast-exchange Ral mutants in tumor cell lines are sufficient to induce STAT3 phosphorylation. STAT3 is constitutively activated in a number of human cancers and possesses oncogenic potential (Bromberg et al., 1999). Inhibiting STAT3 expression in tumor cell lines reduces their anchorage-independent growth to the same extent as inhibiting RalA expression. No additive effect is observed

when we inhibit both RalA and STAT3 expression. Interestingly, inhibiting STAT3 expression also rescues the apoptotic phenotype caused by loss of RalB. These results together suggest that RalA regulates anchorage-independent proliferation through regulating STAT3.

So far, our data is revealing mechanisms by which RalA and RalB proteins generate a minimal oncogenic platform. RalB and Sec5 are mediating tumor cell survival whereas RalA and STAT3 contribute to anchorage-independent proliferation. More experiments will be carried out to understand how the regulation occurs.

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